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Original Article

Development of RT-PCR Using External and Internal Positive Controls Based on 5' Untranslated Region (UTR) for Molecular Detection of Avian Infectious Bronchitis Virus

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Abstract

Background and Aims: Infectious bronchitis virus (IBV) belongs to the group of gamma coronaviruses along with other avian coronaviruses. The disease caused by IBV can appear similar to infectious laryngotracheitis, avian influenza, and velogenic Newcastle disease, which are high priority diseases. The clinical signs can be accompanied by mortalities in broiler chickens and reduced eggshell and albumin quality in layer hens, leading to economic loss for the poultry industry. Rapid detection of IBV is useful for implementation of control measures, research purposes, and understanding the epidemiology and evolution of IBVs. The aim of the present study was the rapid identification of IB with the molecular method, which targets the 5' untranslated region (UTR) gene of IBV that is less variable than the other genes, with homologies greater than 90% among IBV strains.

Materials and Methods: The primers designed to amplify a conserved fragment of the gene. Analytical sensitivity and specificity of the assay were determined.

Results: The results of specificity exhibited the specific amplification of the designed primers for IBV. Sensitivity was 10 pg/µl of the pTZ57R/T-5' UTR. This is the first report of RT-PCR method coupled with construction of comparative internal positive control (IPC) according to 5′UTR gene for accurate detection of IBV. 100 fg/µl of the IPC amplified in the presence of the limit of detection (10 pg/µl) of 5' UTR gene was determined as the optimal concentration of IPC plasmid for RT-PCR of clinical specimens.

Conclusions: The RT-PCR assay presented provides a time saving, sensitive, and reliable method for detection of IBV.

Keywords: Avian Infectious Bronchitis Virus, Internal positive Control, Detection, RT-PCR

Introduction

Infectious bronchitis virus (IBV) is a Gammacoronavirus, a member of the Coronaviridae family (1). It is an enveloped, positive-sense, single-stranded RNA virus, and is highly contagious and prevalent in all countries with an intensive poultry industry, affecting the performance of both meat-type and egg-laying birds (2). IBV belongs to the group of gamma coronaviruses along with other avian coronaviruses. It is a positive-stranded RNA virus, with a genome of about 27 kb containing 5′ and 3′ untranslated regions (UTRs) with a poly (A) tail (3).

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A major part of the genome is composed of two overlapping open-reading frames (ORFs), 1a and 1b, which are translated into large polyproteins 1a and 1ab, respectively, through a ribosomal frame shift mechanism. The primary structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N) are coded by regions found in the remaining part of the RNA genome (4, 5).

IBV is the causative agent of avian infectious bronchitis (IB), which is characterised by respiratory and/or renal and/or reproductive disease (6). The disease caused by IBV can appear similar to infectious laryngotracheitis, avian influenza, and viscerotropic velogenic Newcastle disease, which are high priority diseases (7).

The clinical signs can be accompanied by mortalities in broiler chickens and reduced eggshell and albumin quality in layer hens, leading to economic loss for the poultry industry and farmers. In broilers, IBV affects weight gain and feed efficiency, and, when complicated with bacterial infections like E. coli or S. aureus, it causes high mortality and increased condemnations (8-10).

Many different IBV strains have been isolated and classified around the world that are extremely difficult to control because multiple serotypes and variants of the virus occur that are not cross-protective (11, 12). So, rapid detection of IBV is useful for implementation of control measures, research purposes, and understanding the epidemiology and evolution of IBVs (13, 14).

Various vaccines and protocols to control this viral disease have been developed, although the severity of this disease varies from place to place and flock to flock (15). Live attenuated vaccines for the major serotypes of IBV can be used to control most outbreaks, but it is necessary to isolate and definitively identify the serotype of the virus responsible for the outbreak (16).

Several laboratory methods such as virus isolation in Embryonated chicken eggs (ECEs) , the reference standard and organ cultures and serological tests are available for detecting and differentiating avian viral respiratory infections (17-19). However, these methods are time - consuming and laborious and may require more than one passage before obtaining a result (20, 21). Nevertheless, molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR) are rapid, specific, and accurate (22-25). Compared to virus isolation, these assays may lack sensitivity when used directly from clinical specimens. To overcome this drawback, viral RNA extraction is preceded by the propagation of the virus in chicken embryonated eggs for 36–48h (26, 27).

The aim of the this study was the rapid identification of IBV with the molecular method. The 5' UTR gene was chosen since it is highly conserved among IBV strains and is abundant in infected cells. In this study, an RT-PCR targeting the 5' UTR gene for IBV detection was developed.

Methods

cDNA synthesis. IBV vaccine (Massachusetts type, H120, Merial) was candidate for PCR and preparation of positive control. CinnaPure One kit (sinaclon , Iran) was selected for total RNA extraction from the candidate vaccine using manufacture's instruction. Quality and quantity assessment of the extracted RNA was carried out by optical absorption measurement. Random hexamer primer was applied for cDNA synthesis according to the standard protocol.

Primer design for external positive control construction. IBV 5' UTR genes were chosen for Primer Design using primer CLC work-bench version 3.5.1 (CLC Bio, Denmark).

The forward primer sequence was 5′ GGCT-ATACGACGTTTGTA 3′ and the reverse one was 5′ GGAGATACTCCCTGT-TTTAG 3′. Primer synthesis was done by Bioneer Co. These primers amplified a sequence of 129 bp. RT-PCR was performed at a volume of 25µl including dNTPS (0.2 mM), MgCl2 (1.5 mM) and 0.4 mM from each of the primers, one unit of the Taq DNA polymerase enzyme and 50 ng of cDNA . The negative control reaction was set as a reaction similar to the above but with deionized water instead of cDNA. Thermal conditions of the PCR consisted of primary

denaturation at 94 ° C for 2 minutes, 34 cycles of denaturation at 94 °C for 30 seconds, annealing at 45 °C for 30 seconds, amplification at 72 °C for 35 seconds.

Specificity determination of the RT-PCR assay. For determination of the specificity, PCR reactions were done according to above conditions in the presence of 50 ng of other virus's genomes such as Newcastle disease virus (B1 Type, LaSota Strain) , Avian influenza virus (H9N2), infectious laryngotracheitis S(Vaccine),Infectious bursal disease (IBDL vaccine) as a negative controls. In addition, 50 ng of cDNA of IBV used a positive control.

Production of external positive control. For preparing external positive control, the PCR product was first purified using PCR purification kit (Bioneer, Korea). Ligation reaction between purified sequence and pTZ57R/T vector was done using T4 DNA ligase (Fermentas, Canada) at 22° C for 2 hours. The ligated construct was transferred to the competent E.coli JM107 cells.

The recombinant cells were cultured on Luria Bertani Agar (Merck, Germany) including 5-bromo-4chloro-3-indole beta di-galactopyranosid (X-Gal) with 40µg/ml, isopropyl beta-D-thiogalacopyranoside (IPTG) with a concentration of 38.4 µg/ml, ampicillin with concentration of 100 µg/ml and Nalidixix acid with the concentration of 30 µg /ml. The mentioned culture was incubated at 370C for 16 hours.

PCR on colony assay was done for selection of desired recombinant white colony. Plasmid extraction was made via AccuPrep Plasmid Mini Extraction Kit (Bioneer, Korea). Final confirmation of the insert-receiving plasmid (pTZ57R/T-5' UTR), the restriction map of the plasmid was appraised. For depiction of the map, 500ng of pTZ57RT-5' UTR plasmid was digested with 2 units of the enzyme XbaI (Fermentas, Canada).

Determination of sensitivity. In order to determine the sensitivity, 100 ng/ul of pTZ57RT-16S plasmid were serial diluted (1µg, 100ng, 10ng, 1ng, 100pg and 10pg).

PCR assay was performed on all serial dilutions. The last dilution of the pTZ57RT-5' UTR plasmid yielded a detectable band on the agarose gel was assigned as the limit of detection (LOD).

Production of the internal positive control. The pTZ57R/T-5' UTR plasmid was used to create an internal positive control in accordance with study by Majidzadeh et al (2014) using CLC Workbench version 3.5.1 (CLC Bio, Denmark) (28). Briefly, based on the amplicom of diagnostic primers of IBV (anneal to the oligonucleotides No.1-18 (forward primers) and No. 110-129 (reverse primers). Consequently, an inner forward with MscI site at 5' end (5´ TGGCCAGTTTGTAGGGGGTAGTGCC 3´, anneals to nucleotides No. 12-30) and an inner reverse primer MscI site at 5' end (5´ TGGCCACCCTGTTTTAGGCTTGAAG 3´, anneals to nucleotides No. 102-120) was designed. The PCR reactions were prepared according to the standard protocol in total volumes of 25 µl with annealing temperature of 50°C. An inner forward primer was amplified a 118 bp fragment 1 together with diagnostic reveres primer of IBV.

An inner reverse primer was amplified a 120 bp fragment 2 together with diagnostic forward primer of IBV. Fragment 1 and 2 were cloned in E.coli JM107 according to manufacturer's instruction (Fermentas, Canada). The colonies receiving each of the fragments 1 and 2 were chosen by colony PCR. Plasmid extraction was done separately using AccuPrep Plasmid Mini Extraction Kit (Bioneer, Korea). Finally, the confirmed plasmids were separately linearized with MscI enzyme based on standard protocol (Fermentas, Canada).

Concerning the complementary sites which presences in two fragments; a ligation reaction was performed between the two linearized fragments by T4DNA ligase (Fermentas, Canada). The ligation product was cloned in the E.coli JM107. Screening of the desired recombinant colony (Containing fused fragment 1 and 2) was carried out with colony PCR using diagnostic forward and reverse primers of IBV. Final confirmation of IPC plasmid was applied by Cycle sequencing method using universal primers. The sequencing results were checked by CLC Workbench version 3.5.1 (CLC Bio, Denmark).

The best IPC concentration. To determine the best concentration of the IPC plasmid to apply for detection of clinical specimens, the following research was utilized. Non-infected tracheal or cloacal swabs samples were spiked with a 10-fold serial dilutions ranging 10 ng to 10 fg/µl of IPC plasmid. Total RNA was extracted on specimens by a QIAamp® DNA Mini Kit (Qiagen, USA). Diagnostic PCR assay for molecular identification of IBV was done with diagnostic IBV primers in the presence of extracted IPC plasmid and 10 ng (equal to the limit of detection of the method) of pTZ57R/T-5 UTR plasmid.

Results

Analytical specificity of the RT-PCR assay. RT-PCR assay with diagnostic primers of IBV 5' UTR showed 129 bp band. Analysis of the agarose gel related to electrophoresis of the PCR on negative control viruses' genomes using IBV 5' UTR diagnostic primers exhibited no amplification. These results emphasized the specificity of the assay for IBV (Figure 1).

Analytical sensitivity of the RT-PCR assay. PCR amplification of the recombinant plasmid containing IBV 5' UTR cDNA of IBV and digestion process confirmed the existence of desired gene in pTZ57R/T vector. Sensitivity determination confirmed 10 ng/ul concentration was the last amplifiable dilution of the pTZ57R/T-5' UTR. Hence the LOD was determined 10 ng/ul (Figure 2).

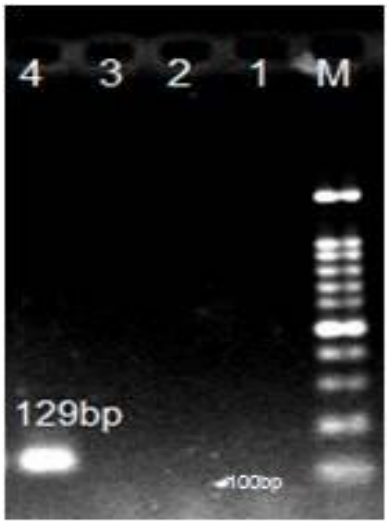

Fig. 1. The results of specificity determination of the assay. M: Marker 100 bp; 1: Negative control; 2: No amplification of Newcastle disease virus (B1 Type, LaSota Strain); 3: No amplification of Avian influenza virus (H9N2); 4: 129 bp band of IBV 5' UTR.

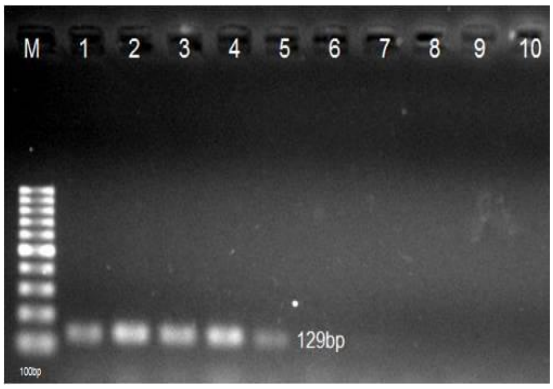

Fig. 2. The results of the PCR on serial dilution of external positive control. These data showed LOD of the assay equal to 10 pg/µl.

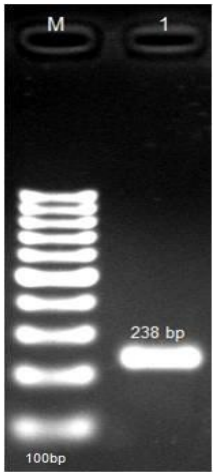

Fig. 3. The specific amplification of recombinant IPC plasmid showed 238 bp band.

Table 1: The consequences of amplification of IPC plasmid in the presence of IBV target gene. The results exhibited the best concentration of IPC plasmid for used as an internal positive control equal to 100 pg/ul

Concentration of target gene	Concentration of IPC	Amplification of target gene	Amplification of IPC gene
10 pg/ul	10 ng/ul	-	+
10 pg/ul	1 ng/ul	-	+
10 pg/ul	100 pg/ul	-	+
10 pg/ul	10 pg/ul	-	+
10 pg/ul	1 pg/ul	-	+
10 pg/ul	100 pg/ul	+	+
10 pg/ul	10 pg/ul	+	-

Internal positive control construction. Expected sizes 120 bp and 118 bp after PCR amplification of recombinant plasmid containing fragment 1 and 2 approved the existence of the mentioned products in pTZ57R/T plasmid (data not shown). Enzymatic digestion of the both fragments with MscI enzyme showed linear form of the both plasmid with predicted sizes of 3006 bp and also 3004 bp (data not shown). The colony receiving plasmid containing the complete sequence of IPC was identified by PCR using diagnostic primers of IBV. Amplification of IPC piece showed a band of 238 bp (Equal to the total of the first and second fragments sizes) (Figure 3). The resulted piece was longer than the target gene while both end having similar attachment sites to the target gene. Sequencing of recombinant plasmid containing internal positive control piece and RFLP analysis demonstrated the presence of MscI enzyme in the IPC sequence, as expected. The minimum concentration of the IPC piece, which didn't obstruct in the RT-PCR ampli-fication of the target gene, was introduced as the best concentration. Based on table 1, 10 fg/ul of the IPC amplified in the presence of the limit of detection (10 ng) of 5' UTR gene was determined as the best concentration of IPC plasmid for RT-PCR of clinical speci-mens.

Discussion

Viral respiratory diseases are common causes of economic losses in poultry industry (29). These diseases cause reduction of growth rate and production, high rate of death, prevention and treatment costs (30). Quick detection and differentiation of causative viruses can play an important role in controlling these viruses (31). IBV and NDV are the viruses that frequently affect the respiratory tract of chickens (32). There are several clinically similar viral diseases that can occur in intensive poultry

production and require laboratory differential diagnosis (33). Coronavirus came into the searchlight when the SARS pandemic occurred in 2003. The intense worked spurred then has revealed an unexpected variety of CoV in various host species (34). The high complexity and genetic diversity of coronaviruses and the economic losses the viruses generate in breeding animals justify efforts to develop broad-spectrum detection techniques.

In addition, such assays would be relevant to identify animal reservoirs of new pathogenic coronaviruses (35). Because of high sensitivity and specificity molecular method that conventional methods offer since its introduction researchers use it extensively as an indispensable diagnostic method to detect viruses (13, 36).

In this study, we developed an RT-PCR targeting a conserved region of the coronavirus 5´UTR gene developed. Targeting the 5´UTR gene has been postulated to be a better choice to improve the sensitivity of the RT-PCR as this gene is supposed to have the most abundant copy number during viral replication (37, 38). The RT-PCR assay described in this study targets the 5´UTR gene of IBV, which is less variable than other genes, with homologies greater than 90% among IBV strains (39).

The primers designed to amplify a conserved fragment of the gene. It is important to rapidly differentiate IBV from disease agents like highly pathogenic avian influenza virus and exotic Newcastle disease virus, which can be extremely similar in the early stages of their pathogenesis (40). Accordingly, the specificity of our test was demonstrated by the absence of positive reaction with other RNA viruses Newcastle disease virus (B1 Type, LaSota Strain), Avian influenza virus (H9N2), infectious laryngotracheitis (Vaccine), Infectious bursal disease (IBDL vaccine) and confirmed negative for coronavirus infection. Based on serially diluted cDNA standards, the RT-PCR detected down to 10 ng/ul of cDNA from IBV.

Adzhar et al (1996) developed universal oligonucleotides for the detection of IBV by the PCR. RT-PCR analysis using these oligonucleotide pairs showed that a number of field isolate preparations also contained a small amount of Massachusetts (Mass) serotype virus, probably of vaccine origin and indicative of low-level persistent infection (41).

Callison et al (2006) developed a real-time Taqman RT-PCR assay for the detection of IBV from infected chickens. The assay amplifies a 143-bp product in the 5' UTR of the IBV genome and has a limit of detection and quantification of 100 template copies per reaction. All 15 strains of IBV tested as well as two Turkey coronavirus strains were amplified, whereas none of the other pathogens examined, tested positive (40). Escutenaire et al (2007) designed RT-PCR assay based on SYBR Green chemistry and degenerate primers were developed for the generic detection of coronaviruses.

The primers, designed in the open reading frame 1b, enabled the detection of 32 animal coronaviruses including strains of canine coronavirus, feline coronavirus, transmissible gastroenteritis virus (TGEV), bovine coronavirus (BCoV), murine hepatitis virus (MHV) and IBV (42).

Shirvan & Mardani (2014) reported that the duplex-RT-PCR is a quick and sensitive procedure for simultaneously detecting IBV and NDV in birds with respiratory infections. They designed their RT-PCR reaction based on 3UTR of IBV and M gene of NDV (43). In addition, high-resolution melt curve analysis (HRM) based on 3 UTR region can rapidly assign the majority of IBV present in field submissions to known subgroups. Importantly, HRM curve analysis also identified variant genotypes that require further investigation (44). PCR primers were designed to conserved regions within the nucleocapsid (N) gene of TCV and to the polymerase gene of TAstV-2. A multiplex RT-PCR assay was developed for the simultaneous detection of two enteric viruses of poultry: turkey enteric coronavirus (TCV) and turkey astrovirus (TAstV). PCR primers were designed to conserved regions within the N gene of TCV and to the polymerase gene of TAstV-2. The detection limit was determined to be 10 ng of RNA used as starting template (45).Meir et al (2010) designed a real-time TaqMan RT-PCR assay

for the detection of IBV in chickens and compare it with RT-PCR and virus isolation. In that study, the development of a real-time TaqMan RT-PCR targeting the highly conserved N gene of IBV and including an internal PCR control is described. Real-time RT-PCR and virus isolation were 17–75% more sensitive than RT-PCR targeting the S1 gene for testing tracheal swabs directly from experimentally infected chicks. When tracheal and cloacal swabs from clinical specimens were tested directly, 50% more samples were positive by real-time RT-PCR than by the S1 gene RT-PCR (39). Acevedo et al (2013) evaluated a duplex SYBR Green I-based real-time RT-PCR assay for the simultaneous detection and differentiation of Mass. and non-Mass. serotypes of IBV. The detection limit of the assay was 8.2 gene copies/mL based on in vitrotranscribed RNA and 0.1 EID50/mL. The assay was able to detect all the IBV strains assessed and discriminated well among the IBV Mass. and the IBV non-Mass. serotypes strains (46). To our knowledge, this is the first report of an RT-PCR method couple with constriction of comparative IPC according to 5´UTR gene for accurate detection of IBV.

The addition of an internal positive control for the detection of PCR-inhibitors allows the distinction between true and false negatives. Various PCR inhibitors such as EDTA, heparin, hemoglobin, ethanol and SDS may not remove during DNA extraction procedure and lead to reaction inhibition. Therefore, a strategy to provide the necessary information for accurate interpretation of PCR assay results is required (47, 48). To monitor the false negative results of PCR, different standards have been developed (49). According to the guidelines of international standard organization (ISO), the presence of internal positive control (IPC) in the reaction is mandatory (28, 50).

IPCs are classified as competitive and noncompetitive. Noncompetitive IPCs include a group of internal controls with primer annea-ling sites different from target gene (28). It is recommended to use competitive IPC instead of a noncompetitive IPC. Competitive IPCs have similar primer annealing sites for the target gene and are amplified by the same primer pairs and conditions along with the target gene. The method proved to be suitable for diagnostic purposes, showing high sensitivity and specificity when applied to biological samples from different animal species (51).

In conclusion, the RT-PCR assay presented in this paper provides a time-saving, sensitive, and reliable method for detection of IBV directly from tracheal or cloacal swabs, as well as in allantoic fluid from infected embryonated eggs.

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